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Molecular approaches for forensic cell type identification: On mRNA, miRNA, DNA methylation and microbial markers

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ABSTRACT

Human biological traces have the potential to present strong evidence for placing a suspect at a crime scene. In cases, the activity that led to deposition of an individual's cellular material is increasingly disputed, for which the identification of cell types could be crucial. This review aims to give an overview of the possibilities of the employment of mRNA, miRNA, DNA methylation and microbial markers for tissue identification in a forensic context. The biological background that renders these markers tissue-specificity is considered, as this can affect data interpretation. Furthermore, the forensic relevance of inferring certain cell types is discussed, as are the various methodologies that can be applied. Forensic stains can carry minute amounts of cell material that may be degraded or polluted and most likely cell material of multiple sources will be present. The interpretational challenges that are imposed by this compromised state will be discussed as well.

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1. Scope of the review

Within the forensic sphere, propositions can be placed in three generic classes that centre on source, activity or offence level [1,2]. Typical questions at these three hierarchical levels are: "Could the semen present in the evidentiary stain have originated from the defendant?" (source level), "What actions resulted in the observed bloodstaining pattern?" (activity level), "Is the defendant guilty of the crime?" (offence level). Clearly, offence level questions are the duty of the court and not the forensic expert. Source level questions have been the main focus of human forensic genetic research during the last decades. Step by step, the sensitivity of DNA analyses improved and human genetics advanced from comparative analysis to surveying phenotypic and ethnic traits of perpetrators [3]. The activity that led to the deposition of the cellular material is more and more assessed [4,5]. Growing societal forensic awareness, increasing appeals to "the right to remain silent" and contradictory testimonies have stimulated this. The investigation of fingerprints and bloodstain pattern analysis can assist evaluation at the activity level. Moreover, knowledge regarding the cell types residing in an evidentiary trace can facilitate inference of activities. Body fluids such as blood, saliva, semen, vaginal mucosa and menstrual secretion are mostly assessed, but skin or organ tissues (such as brain, lung, kidney, liver, heart and skeletal muscle) are also examined. In recent years, molecular approaches for cell type inference have been intensively researched following various strategies. This review will describe and discuss biological, forensic and technical aspects for four marker types, namely expression of messenger RNAs (mRNAs) and microRNAs (miRNAs), determination of DNA methylation levels and the identification of microbial species. Proteomic biomarkers [6–8] are not included in the review, as these do not demonstrate sufficient reproducibility among laboratories.

2. Biological background of molecular markers

2.1. Messenger RNAs

An adult human body contains around 210 distinct cell types specialised for different functions [9]. Regardless of cell type, cells all possess the same diploid genome, except for the haploid gametes and anucleated cells such as platelets, red blood and squamous cells. Forensic DNA research relies on this basic principle, as DNA profiles of for instance bloodstains are compared to those derived from buccal reference swabs. Different cells can, however, have very different physical characteristics regarding size, shape, metabolic activity and responsiveness to signals. During embryonic development the determination of cell type is highly controlled and occurs at the level of gene expression, which commences with the transcription of DNA into RNA and will

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proceed to translation to proteins when coding RNAs are concerned (ribosomal and transfer RNAs are not translated). Spatial and temporal differences in gene expression derive from combinatorial interactions between transcription factors plus the effects of chromatin state or enhancer/silencer regions in the DNA. As a result, each specialised cell type expresses only a subset of the 22,000 coding genes that constitute the full human genome and this mRNA set serves as the chemical blueprint for the production of proteins that present the phenotypic manifestations of genes. A fully processed mRNA is spliced (a mature human mRNA has on average 9 exons-retained sequences- and 8 introns-removed sequences-), includes a stabilising 5′cap (7-methylguanosine RNA), a 5′-UTR (untranslated region; typically 150 nucleotides (nt)), a coding region, a 3′-UTR (average length 500 nt) and a poly(A) tail (around 200 nt) [9]. Messenger RNAs have a limited lifetime, with an average half-life of 10 h for human mRNAs [9,10]. This allows a cell to alter protein synthesis rapidly in response to its changing needs. Accordingly, decay rates are generally relatively low for housekeeping mRNAs and relatively high for mRNAs coding transcription factors [11].

While only 2% of the genome is translated into proteins, at least 85% is transcribed [12]. Probably not all transcribed RNAs will be functional RNAs, as they are the result of pervasive transcription (transcription beyond the boundaries of genes, resulting in non-coding intergenic RNAs), spurious transcription starting wherever RNA polymerase binds the genome or read-through transcription (imperfect termination) [12]. Cells have surveillance systems in place to ensure fidelity and quality of mRNAs; mRNAs with premature stop codons are degraded by nonsense-mediated decay [13] and double-stranded RNAs induce RNA silencing [14]. Both by microarray analysis (hybridisation to spotted probes) and by RNA-Seq (whole transcriptome shotgun deep-sequencing), RNA expression level information has been obtained, and tissue-specific mRNA profile information has been collected in expression portals such as GTEx [15] that is based on RNA-Seq data, and BIOGPS [16] that uses microarray data. Interestingly, low-level expression in many tissues is evident for many mRNAs, and tissue-specific mRNA expression appears not to be absolute.

2.2. Micro RNAs

Regulatory RNAs and especially miRNAs also contribute to cell type differentiation [17]. miRNAs belong to the class of small regulatory RNAs and are produced from specific genetic loci that have evolved to regulate mRNAs. Currently, over 1800 human miRNAs have been registered [18]; several have high expression levels, some show resemblance to each other and are placed in miRNA families and others appear evolutionarily conserved (either ancestral- or lineage-specific) [19]. Mature miRNAs have a length of around 22 nucleotides, associate with a protein of the Argonaute class (which is a specialised family of RNA-binding proteins with an endoribonuclease domain) and base pair to mRNAs (primarily their 3′-UTR) to establish mRNA decay or inhibition of translation [20]. In some species such as plants, miRNAs predominantly fully base-pair with their mRNA targets, whereas human miRNAs generally interact only through limited base pairing for which seven nucleotides in the 5′ part of the miRNA (the so-called seed region, nt 2 to 8) are most important [21]. Since only a few bases suffice for mRNA–miRNA interaction, a miRNA can have several, up to hundreds, of targets [21]. Conversely, an mRNA may be targeted by several (different) miRNAs, either because there are multiple miRNA binding sites in the 3′UTR or because different miRNAs share the same binding motif. With imperfect base-pairing, the gap-based interaction establishes translational repression and accelerated mRNA deadenylation; with perfect base pairing, RNA cleavage is induced as the scissile phosphate of the RNA target is now close enough to the endoribonuclease cleavage site of the Argonaute–miRNA complex. The genes encoding miRNAs or miRNA clusters are located within introns, intergenic regions and exons, and thus often expressed co-transcriptionally, under control of common regulatory sequences. This can result in tissue-specific expression patterns [22]. Like mRNA expression, miRNA expression may not be fully cell type-specific because of background transcription.

2.3. DNA methylation

In addition to variations at the mRNA and miRNA level, tissue-specific differences occur in the epigenome; the network of chemical compounds that surrounds the DNA and affects gene expression. The epigenome includes DNA methylation, histone modification and chromatin structuring. Only DNA methylation marks (a methyl group at the 5′ position of cytosine residues) remain in extracted DNA, so this epigenetic mark is best compatible with standard forensic procedures. DNA methylation has a role in embryonic development, reprogramming, transcription, chromosomal stability (including repression of transposable elements), imprinting and X-chromosome inactivation [23]. DNA methylation generally relates to transcriptional suppression and can recruit methyl-CpG-binding domain proteins and chromatin-remodelling proteins such as histone deacetylases that govern heterochromatin formation [24]. Although DNA methylation can be retained through mitosis, it is not a static feature and it is subject to changes induced by age and various environmental factors, such as diet and perceived stress [23].

Cytosines in CpG dinucleotides are the preferred substrates for DNA methyltransferases and 70% to 80% of all CpGs (around 28 million in total) are methylated in humans [25]. The abundance of CpG dinucleotides in human DNA is lower than expected based on the GC content, due to the inherent mutability of methylated cytosine (methylated cytosine can be deaminated and become thymine). Unmethylated CpGs are often grouped in clusters called CpG islands, which are GC-rich (>55%) regions of at least 500 base pairs at or near the transcription start site of genes with an elevated number of CpGs (observed/expected ratio CpG >65%) [26]. CpG islands act as promoters and about 50% of the human genes have a CpG island promoter. Differentially methylated regions (DMRs) occur between tissues, developmental stages and cancer/normal samples [27]. Differential methylation between tissues, occurs in both promoter-associated regions and in gene bodies (transcription units) [28]. Methylation at gene bodies appears to associate with reduced transcriptional noise (variability in gene expression) [29]. Regarding the promoter-associated regions, the greatest differential methylation is not at CpG islands but at CpG island shores [27], which are the sequences up to 2 kb distant from CpG islands. When relating the levels of gene expression and methylation in the CpG island promoter areas, an inverse correlation was found, supporting the hypothesis that hypo-methylation (decreased methylation intensity) and not hyper-methylation (increased methylation density) is associated with tissue-specific functions [28].

2.4. Microbial species

An adult human body consists of approximately 40 trillion human cells [9]. In addition, around ten times more microbial cells are present, that account for only 1–3% of the total body mass due to their much smaller size [9]. Microbes comprise bacteria (that are prokaryotes), fungi, algae and parasites (all eukaryotes) and viruses and prions (considered akaryotic) [30]. The human microbiome is extensively studied by a global initiative known as the Human Microbiome Project [31–33]. Microbes reside on

surfaces in- and outside the human body including skin, mouth, intestines and vagina to support food digestion and/or secure human health. Changes in the human microbiome are related to health issues such as periodontitis, obesity, inflammatory bowel diseases and vaginosis [34,35]. Different body sites have distinctive microbial communities, although the exact bacterial makeup for a given site varies between individuals with a possible further variation within the individual; hands belonging to the same person can show different bacterial fingerprints [36]. The body sites with the greatest commonality among individuals are the oral sites, followed by nose, stool, skin and finally vaginal sites [37]. Still, a core microbiome or generic microbial species may be absent as was shown for vaginal samples [38].

Microbes can be transferred and may remain at surfaces contacted by humans, for which restroom surfaces are well known [39]. Furthermore, microflora residing in a body cavity may transfer to proximate locations or body sites that can have contact (e.g. vaginal flora may transfer to groin or penis) [38]. The abundance and persistence of the species may be less at these secondary locations.

3. Forensic relevance of identifying stains

3.1. Stain detection

The analysis of stains starts with their detection. Hairs and bloodstains are generally visible to the naked eye although on dark fabrics bloodstains can remain unnoticed. To enhance contrast, light of discrete wavelengths can be applied building on the inherent luminescent properties (absorption or fluorescence) of body fluids [40]. Alternatively, scenario-based evidentiary sampling is performed such as for sexual assault kits and with the recovery of contact traces based on, for instance, victim statements. Another option to locate evidence not visible to the naked eye is the use of presumptive tests, which are generally based on chemical and catalytic reactions such as with luminol enhancement of latent bloodstains and α-amylase testing for saliva [41]. These presumptive tests give an indication regarding the identity of the sample and can be followed by confirmatory assays that have a smaller risk of false positive results. Examples of confirmatory tests are microscopic examination for sperm cells and lateral flow tests detecting human antigens such as the prostate specific antigen present in seminal fluid [41]. A limited number of body fluids can be assessed using these methods namely semen, saliva, blood and urine. The identification may not be unequivocal as for instance α-amylase occurs not only in saliva but also in pancreas from where it can reach the colon and mix with faecal material [42], while blood cannot be discriminated from menstrual secretion. The need for more specific assays that allow discrimination of more body fluids and tissue types, preferably by human-specific tests, is therefore evident.

3.2. Violent assaults

Violent assaults generally evoke the presence of blood on evidentiary items. Peripheral blood can derive from cuts and impacts that result in direct or indirect depositions. Expirated blood (blood from an internal injury mixing with air from the lungs and expelled through the nose and/or mouth) that matches the DNA profile of a victim on the clothing of a suspect can suggest that the suspect was in the vicinity of the victim during the crime. A nasal bleed can result from a thump on the nose or occur spontaneously, and depending on the statements in a case nasal blood is indicative of an assault or serves as an alternative inoffensive scenarios. Likewise, menses may be proposed as an alternative inoffensive scenario for a bloodstain in an alleged violent assault. Bloodstain pattern analysis may assist the interpretation (an expirated spatter tends to form a fine mist, for instance [43]), but it can be useful to complement staining pattern analyses with data from molecular markers that may derive from the different compositions of the various blood-containing body fluids. Expirated blood represents a mixture of peripheral blood and saliva and/or nasal secretion. Saliva is secreted by the salivary glands and includes mucus, digestive enzymes and oral microflora. Nasal secretion is generally composed of mucus, plasma leaking from blood vessels, tears seeping through the nasolacrimal duct and nasal microbiota [44]. Nasal blood can be considered a mixture of peripheral blood and nasal secretion. Menstrual secretion contains peripheral blood, old parts of uterine tissue, cells from the mucus lining of the vagina,

Table 1
Overview of body fluids and tissues that can be encountered at a scene of crime in combination with marker types that have been described for these body fluids and tissue. Details and references follow in Sections 4.4–4.7.

	Type	Forensic relevance	Marker type			
			mRNA	miRNA	Meth	Microbial
Body fluids	Blood	Violence, human-specific assay	x	x	x	
	Semen, fertile	Sexual assault, confirmation sampled area	x	x	x	
	Semen, sterile	Sexual assault, confirmation sampled area	x	x		
	Saliva	Sexual assault e.g. licking, kissing or inoffensive stain	x	x	x	x
	Vaginal mucosa	Sexual assault, confirmation sampled area	x	x	x	x
	Menstrual secretion	Sexual assault or inoffensive alternative scenario	x	x	x	x
Touch	Skin	Confirmation sampled area	x	x	x	x
Other secretions	Expirated blood	Violence, confirmation bloodstain pattern analysis				x
	Nasal blood	Thump on the nose or inoffensive alternative scenario	x			
	Nasal secretion	Inoffensive alternative scenario	x			
	Sweat	Confirmation witness report, cross-reactivity?	x			
	Urine	Confirmation sampled area, cross-reactivity?	x			
	Tears	Possible inoffensive scenario if cross-reactive				
	Breast milk	Possible inoffensive scenario if cross-reactive				
	Vomit	Contains saliva and stomach content, inoffensive scenario				
	Faeces	Anal sexual assault				x
Organs	Brain	Head injury	x	x		
	Heart, lung	Chest injury	x			
	Kidney, liver	Abdominal injury	x			
	Skeletal muscle	Injury	x			

matrix degradation factors involved in the onset of menses (e.g. matrix metalloproteins) [45] and bacteria making up the vaginal flora. From the description of these complex body fluids, it follows that both endogenous and microbial markers may be of use to infer body fluid identity (Table 1). A complication with complex body fluids may be their discrimination from superimposed 'simple' fluids. Besides the staining pattern, the distribution within the stain may be informative as in superimposed stains unequal dispersal is expected.

Violent assaults may also evoke the presence of organ tissues such as brain, heart, lung, kidney, liver or skeletal muscle. Inferring organ tissue residing on bullets, implements, clothing or surroundings (floor, wall etc.), may be useful for crime reconstruction. Shooting reconstruction may inform on the distance of the shooter from the target, the path of the bullet(s), the number of shots fired, the sequence of multiple discharges and more. As with expirated blood, the presence of for instance brain material matching the DNA profile of a victim on the clothing of a suspect can suggest that the suspect was in the vicinity of the victim during the crime. Up to now, only RNA markers have been implicated in organ type inference (Table 1) [46,47].

3.3. Sexual assaults

Sexual assaults deliver different types of evidence in which the detection of semen/seminal fluid, saliva, vaginal mucosa, menstrual secretion or faeces may be informative for the dispute of events. Semen is a mixture of spermatozoa and fluids from the seminal vesicles, the prostate and the bulbourethral glands. For fertile males, spermatozoa markers can be used while the detection of semen from azoospermic males relies solely on seminal fluid markers. When spermatozoa are detected through microscopy, molecular cell typing assays will not present much added value. Sterile seminal fluid has the limitation that a DNA profile may not be obtained due to the absence of spermatozoa, and a DNA-link to a suspect or unidentified perpetrator may not be established. Thus, the forensic value of detecting seminal fluid may be limited to verification of statements. In case of penile swab examination, the presence of semen or seminal fluid can be confirmatory for the sampling location.

With alleged vaginal penetration, the presence of vaginal mucosa is questioned. Mucous membranes line various cavities in the human body such as oral, nasal, anal and vaginal cavities and markers initially identified as saliva or vaginal markers may rather represent general mucosa markers [48]. Alternatively, microbial markers are used as the vagina has a distinct microflora [38,49,50]. This flora may, however, transfer to proximate (groin) or contacting (penis) body sites [38]. Vaginal markers can be used to assess the presence of vaginal mucosa on penile swabs, fingernail dirt, male underpants or objects allegedly inserted. Alternative scenarios for the presence of female cell material on these items may be saliva (vomit), nasal secretion, skin, sweat, urine or tears that were directly or indirectly transferred. Thus, it is important to establish whether these fluids are cross-reactive with the vaginal markers, which has been assessed for some fluids and various levels of cross-reactivity has been reported with saliva [51–54] and nasal mucosa [44,55]. The presence of vaginal mucosa can also only confirm sampling location for instance when female underpants are examined for the presence of saliva in a disputed licking event. In case of an alleged anal penetration, faecal markers may be applied that are generally of microbial origin [56]. Also with these markers cross-reactivity from other body fluids or because of transferred microbiota may occur. With faecal material it will be more difficult to generate a human DNA profile as stool contains few human cells and the value of cell type information may be limited to the assessment of statements.

In specific cases, its presence of menstrual secretions may be helpful to determine timing of intercourse: a woman stated to have been sexually assaulted by a man with whom she had had consensual intercourse a few days earlier. Since she had started menstruating at the time of the alleged assault, the presence of menstrual secretion on the suspect's penile swab supported her claim.

With sexual assault samples, skin may be present either because a skin area was sampled or because of touch contact. Thus, the presence of skin is not in dispute and showing the presence of skin is foremost confirmatory of sampling location. Furthermore, detecting skin may explain alleles matching suspect or victim in the DNA profile, which may be the main reason to include skin markers [46,57–60]. Interestingly, not all skin areas have the same properties regarding the propensity to release DNA: the non-sebaceous areas on the palms of the hands and the soles of the feet give less DNA than sebaceous areas like the face and the scalp [61]. It is proposed that sebum secreted by the sebaceous glands is a more important DNA source than sloughed off keratinocytes [61]. This may imply that DNA on hand palms is actually present through secondary transfer within an individual and derives mainly from touching one's face or scalp. DNA in sebaceous fluid appears mainly single-stranded [61] while the presence of RNA in sebaceous fluid has not yet been examined and is difficult to predict.

Few studies have included sweat markers [55,62]. Sweat deposition is likely linked to skin deposition, and the role of a sweat-specific marker may reside in for instance the confirmation of a sweaty status of a suspect as indicated by an eyewitness or the victim. From the various body fluids generally occurring from inoffensive scenarios (Table 1), only nasal secretion has a high content of human cell material and can make a substantial contribution to a DNA profile. Nasal secretion has been studied and one of the two saliva mRNA markers (STATH) frequently used in forensic assays appears as a strong nasal marker as well [44,55]. To achieve positive identification of nasal secretion while excluding the presence of saliva, it may be useful to identify a true nasal secretion marker. Since tears, urine and breast milk are either less encountered or lower in human cell count, markers for these body fluids seem less urgent, although a urine marker referring to the same gene as one of the kidney markers [46], has been reported [55].

3.4. Forensic stains can be mixtures

From the above scenarios it derives that many samples can present mixtures of various sorts: one donor can contribute multiple cell types (for instance semen and skin on a penile swab), multiple donors can all donate the same cell type (for instance a blood–blood mixture from a fight), multiple donors can contribute different cell types (for instance vaginal mucosa on penile skin) or partly overlapping cell types (for instance saliva and skin from involuntary kissing on skin). It may be impossible to distinguish these scenarios based on test results alone although useful information may derive from the sampling area (skin) or the occurrence of gender-specific body fluids (semen will have had a male and vaginal mucosa a female donor).

4. Forensic application of cell type markers

4.1. Cell types and forensic stains

Section 2 described that specialised cell types carry distinct mRNA/miRNA signatures and DNA methylation profiles, while specific microbial communities inhabit different body sites. A complexity in forensic analyses is that even a single source

evidentiary trace will not translate to a single cell type; many body fluids and tissues are mixtures of various cell types (blood has red blood cells, five types of white blood cells, platelets and progenitor cells). This will not necessarily affect specificity, but may reduce signal strength, as the presence of other cell types will dilute marker responses. With RNA markers, signals will become less strong but generally still discernable as the expressing cell type will have numerous RNA copies; with DNA methylation markers the hypo- or hypermethylation status in a cell type will level out due to the presence of other cell types thereby reducing the discriminatory value of the methylation status (semen is an exception as the main cell type in this body fluid is spermatozoa).

The use of microbial markers has complexity as well; the microbial community at a body site may not straightforwardly relate to the microbes recovered from a crime stain as the composition may alter due to growth or degradation of some species when deposited outside of the human body. These are additional challenges next to the compromised state that forensic evidentiary stains can have. This compromised state refers to sample degradation, contamination by various substances, minute amounts of human cell material or presence of material from multiple donors or cellular origins. The analysis of mixed samples is better possible with a marker type giving "on or off" signals for various cell types than with a marker type that uses a discriminatory value like a methylation rate.

4.2. Nucleic acids from forensic stains

As the standard forensic procedure focuses on DNA extraction for STR typing, RNA-based assays will require additional handling and extended extraction methods. On the other hand, no valuable DNA extract is consumed for cell type inference. When, however, only DNA extract remains in an old case, RNA assays can most likely not be applied [48].

It is most efficient when RNA and DNA extract in different fractions, as RNA extracts are generally freed from DNA to prevent interference by the genomic sequences. Due to ubiquitous ribonucleases, RNA is more prone to degradation than DNA especially in the single–stranded regions not involved in intra-molecular base pairing. Because of fragmentation and when miRNA markers are used, forensic RNA extraction protocols need to include low molecular weight RNA molecules. Although miRNAs receive stability in vivo through the loading into an Argonaute protein (Section 2.2), this protection may be lost ex vivo when a stain is exposed to degrading conditions such as microbial protease activity.

For methylation markers, standard forensic DNA extracts can be used as methyl groups remain on extracted DNA. For microbial markers microbe-specific RNA and DNA assays can be used and these nucleic acids need to be released from the microbe. Microbes are generally harder to lyse than human cells due to the presence of a cell wall and more stringent extraction procedures may apply. This holds especially for gram-positive bacteria that have a thicker cell wall than gram-negative bacteria due to the presence of more peptidoglycan.

4.3. General considerations regarding markers and assays

The development of any assay starts with the selection of suitable markers that-ideally-indisputably discriminate a large panel of body fluids and tissues. To confirm findings and because marker levels may vary between individuals or with physiological condition, it appears favourable to have multiple markers per tissue, irrespective of marker type. Preferably, markers are positive identifiers for a single cell type; it is for instance suboptimal to infer the presence of menstrual secretion from the combined presence of vaginal mucosa and blood markers as this does not discriminate menstrual secretion from a mixture of the two body fluids [63]. Similarly, inferring presence of nasal secretion using a marker that is also expressed in saliva [55] carries the risk that saliva is mistaken for nasal secretion when the second saliva marker drops out (the inference of saliva needs a positive response of both saliva markers and marker dropout may lead to a false identification for nasal secretion). Thus, the nasal secretion marker is not a truly positive identifier. Forensic markers have been derived through literature and databases searches [46,64,65] or by using an analytical approach using (time-wise degraded) samples [57,66].

RNA and DNA-based assays targeting endogenous human sequences have the potential to be human-specific as for most regions (ultra-conserved and some ribosomal sequences excepted [67–69]), genomes show sufficient variation to design species-specific oligonucleotide primers. When using microbial markers, human-specificity is not certain and overlap in for instance the human and mammalian oral microbiome has been reported [70].

Since forensic stains may carry only little cell material, assays need to be sensitive. A generic approach to increase sensitivity is to analyse multiple markers simultaneously in a multiplex assay. These can be markers that assess different cell type and/or multiple markers per cell type. In the next sections, different assay types for the various marker types are described. In Table 2, the advantages and disadvantages of the various marker types are summarised.

4.4. Assays for mRNA markers

Specific mRNA markers have been identified for blood [51,66,71,72], spermatozoa and seminal fluid [51,73], saliva [51,73,74], vaginal mucosa [54,75,76], menstrual secretion [71,74], skin [57,58,60], sweat [55,62], urine [55] and a set of organs that includes brain, heart, lung, kidney, liver and skeletal muscle [46]. Both the sensitivity and the specificity differ for the various candidates, but useful markers are available for all these cell types. For nasal blood and nasal secretion a partially specific marker has been observed [44,77], as one of the frequently used saliva markers is highly expressed in nasal mucosa. Also general mucosa markers are described [48] that can be used to discriminate mucous secretions from blood and semen.

RNA can be analysed by amplification-based methods denoted RT-PCR (reverse transcription PCR) or by hybridisation techniques such as northern blotting, RNase protection assays and digital gene expression methodologies. These latter methods have been developed recently and enable counting of the number of individual transcripts in a sample. A highly sensitive counting method applied for forensic cell type inference uses NanoString® barcode probes that hybridise overnight in solution to extracted RNA or even a crude cell lysate [76,78]. For each target, two specific ~50 nt probes are used; one carries a colour-coded barcode (a seven-spot pattern build from four fluorescent tags), the other facilitates for complex immobilisation that enables counting. Although such digital expression methodologies are highly promising, amplification-based RT-PCR methods are generally better compatible with the equipment available in forensic laboratories and therefore most frequently employed at this moment. RT-PCR commences with the transcription of RNA molecules into complementary DNA (cDNA). Since the mRNA molecules may be fragmented and lacking the poly(A) tail, generally deca- or hexamer oligomers of random sequence are used to initiate reverse transcription. Prior to cDNA synthesis, remnants genomic DNA (gDNA) are removed by DNase treatment to prevent interference. Since no method for human-specific RNA quantification is described, the RNA input for cDNA synthesis is either based on a total RNA quantification result [54,73] (including

Table 2
Overview of advantages (+) and disadvantages (−) of the various marker types.

Marker type	Effect	Feature
mRNA/miRNA	−	Additional procedures: extraction of RNA, DNase treatment and cDNA preparation
	+	DNA extract is not consumed
	−	Cannot be applied when only DNA extract remains in an old case
	+	Mixture analysis possible since markers are present/absent to certain extent
	−	Variable expression: different per marker, influenced by physiological factors
	−	Background signals from spurious transcription
mRNA	+	Multiple candidates per tissue can be readily obtained
miRNA	+	miRNA molecules are in vivo protected against RNases
	+	miRNAs can have high expression levels
DNA methylation	−	Consumes DNA extract (and bisulphite-based assays use large quantities of DNA)
	+	Can be applied when only DNA extract remains in an old case
	−	Often based on differential methylation level: not absence/presence but more/less
	−	Mixture analysis may be difficult as methylation level will dilute
	−	Methylation show inter-person variation and is influenced by age and environment
Microbial	+	Both RNA and DNA-based assay can be used
	+	Mixture analysis possible since markers are present/absent to certain extent
	−	Microbiome various between and within persons, not always a core microbiome
	−	Microflora may change with disease (e.g. vaginosis)
	−	Microbes may not be human-specific and shared by other mammals
	−	Microbiome may transfer to proximate body sites or locations/surfaces in contact

RNA of other species like microbes) or a fixed volume of RNA extract is taken [46,48] assuming that the cDNA synthesis reaction, which generally takes micrograms of RNA, is not overloaded. One could also subject RNA extracts to DNA quantification with the aim to verify that the RNA extract is devoid of gDNA and inhibitors (this information can be derived from the internal positive control).

RT-PCR methodologies group as quantitative RT-PCR (RT-qPCR) or end-point RT-PCR assays, for which products are measured either at each cycle or after completion of all cycles. Consequently, RT-qPCR assays have a wider dynamic range than end-point RT-PCR assays and need no additional procedure to detect the products. RT-qPCR assays can be dye-based (general double-stranded DNA binding) or probe-based (either gain or quenching of fluorescence probes). The multiplexing capacity of RT-qPCR assays is determined by the number of fluorophores and therefore limited. Alternatively, the post-amplification melt data can be used to achieve some multiplexing as each amplimer will have a specific melting temperature based on its sequence and length (denoted high resolution melting analysis, HRM) [79,80]. End-point RT-PCR is generally followed by fragment analysis by capillary electrophoresis (CE), for which greater multiplexing capacity is obtained as markers can be discriminated both by fluorescent label and size. Several large multiplexes to infer forensic cell types have been developed [46,48,55,81]. Since RNA molecules are sensitive to degradation, small amplicon sizes are preferred (between 70 and 150 bp) for all approaches. Preferably, cDNA-specific amplicons are designed making use of the fact that human mRNAs are spliced (Section 2.1). The primers then either cover an intron or one primer is placed over an exon-exon junction. In order to correctly interpret qPCR data, normalisation strategies have to be applied using reference genes, which is challenging as also reference genes may exhibit differences in expression level between cell types. Housekeeping markers are generally also included in end-point RT-PCR assays, but the role is less crucial than in qPCR as it merely indicates the presence of RNA and absence of factors inhibiting cDNA synthesis or PCR, which is especially informative when none of the other markers give a signal.

4.5. Assays for miRNA markers

miRNA markers have been identified for the body fluids blood, semen, saliva, vaginal mucosa and menstrual secretion [82–88], for skin [89] and for brain [47]. The specificity of miRNA markers is found to be generally the best for semen and the worst for saliva and vaginal markers [82–85,87].

As with mRNA assays, RT-PCR analyses are the preferred strategy in forensic miRNA analyses. The small size of miRNAs determines the use of a different reverse transcription procedure than random oligomers, and two general strategies are employed [90,91]. In the first approach the 3′ ends of all miRNAs are elongated using poly(A) polymerase [92]. A primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′ end is then used to prime reverse transcription. In a subsequent RT-(q)PCR, this universal region and a miRNA specific primer are used. The second cDNA synthesis approach does not target all miRNAs but reverse transcribes miRNAs individually using a stem–loop RT primer that consists of a short (e.g. six nucleotides) single-stranded region complementary to the known sequence on the 3′ end of the miRNA and a stem–loop structure [92]. The base pairing in the stem is thought to enhance reverse transcription and reduce spurious priming on larger RNAs [93]. The loop contains a universal primer-binding sequence. Like in the previous approach, the universal region and a miRNA specific primer are used in RT-(q)PCR. Large pools of stem–loop RT primers facilitate simultaneous reverse transcription of multiple miRNAs.

These cDNA synthesis approaches prepare sufficiently long templates for RT-PCR analysis. Mostly, RT-qPCRs are used that require appropriate reference targets for normalisation such as other abundantly and stably expressed small non-coding RNAs like small nucleolar RNAs (snoRNAs guiding chemical modifications of other RNAs) or small nuclear RNAs (snRNAs involved in the processing of pre-mRNA in the nucleus) [84,94]. Sometimes end-point RT-PCR followed by fragment analysis by CE is used to analyse miRNAs [95,96].

4.6. Assays for DNA methylation markers

Highly specific DNA methylation markers have been identified for spermatozoa-containing semen [97–102], while for blood, saliva, vaginal mucosa, menstrual secretion and skin variable results have been obtained [97,100,102–104].

Although direct detection of DNA methylation may be feasible in the future by single-molecule real-time sequencing technologies [105], currently indirect methodologies need to be applied. These

separate into two general strategies that rely on either methylation-sensitive restriction enzymes or treatment with sodium bisulphite. The restriction enzyme approach relies on the presence of an apt recognition site at the CpG of interest. The methylation sensitive restriction enzyme will only cleave this site when the CpG is not methylated. Genomic DNA is subjected to extensive restriction enzyme digestion to ensure all unmethylated DNA is cleaved. Primers flanking the cleavage site will only result in a PCR product with undigested DNA and thus the absence of other unmethylated CpG sites in the same PCR amplicon is fundamental. Multiple amplicons can be amplified simultaneously. The method gives only a signal for the methylated form of the CpG, so it is not possible to infer the percentage methylation that occurs for the CpG (for this also the unmethylated version of the CpG should present a signal). Nevertheless, the relative height occurring for different methylation markers has been used in the context of tissue inference [100]: e.g. when the signal for marker A is higher than that for marker B tissue X is indicated and when reverse tissue Y is indicated. As explained in Section 4.1, most tissues and body fluids comprise several cells types (except semen) and the 'on/off' methylation in one cell type can fade to differential methylation levels in a body fluid or tissue. Methylation-dependent restriction enzymes can selectively cut methylated DNA, but few of these enzymes have been identified, which reduces the chance of a suitable recognition site at the CpG of interest.

Alternatively, DNA is treated with sodium bisulfite that converts unmethylated cytosine to uracil while keeping methylated cytosines unchanged. Uracil becomes thymine in subsequent PCR, and the presence of cytosine or thymine can be detected with various methodologies. PCR products (for which primers have to be specifically designed as all unmethylated cytosines have been converted) can be cloned and sequenced to provide 'methylation heat maps' of individual CpG sites [101]. Methylation specific PCR uses primer pairs specific for unconverted or converted cytosines and methylation rate is determined by the ability of the primers to achieve amplification. In methylation-sensitive single base extensions (e.g. SNaPshot) [100], a primer is annealed just before the CpG of interest and extended with one base after which the ratio of cytosine to thymine is determined. Both the PCR and the extension reaction are readily multiplexed. PCR can also be followed by pyrosequencing, which is a 'sequencing-by-synthesis' method that measures the ratio of cytosine to thymine at an individual site from the amount incorporated nucleotide [102]. qPCR assays (as described in Section 4.4) using fluorescent probes of HRM analysis are also useful to measure methylation levels. Concurrent with conversion, bisulphite treatment invokes the degradation of genomic DNA. Since complete conversion is needed, long incubation times, elevated temperature and high bisulphite concentration may be applied, which can lead to the degradation of about 90% of the incubated DNA. Therefore, bisulphite methods may require more DNA than available in a compromised forensic sample.

4.7. Assays for microbial markers

Specific microbial markers have been identified for oral origin (saliva and expirated blood) [56,106–108], vaginal origin (vaginal mucosa or menstrual secretion) [38,49,50,54,74] and faeces [49,56,109].

The hypervariable regions of the 16S ribosomal RNA gene are generally used to classify and identify microbes [37], as the 16S-rRNA gene is present in most microbes and shows a suitable level of changes. However, primers may target any species-specific region. When RNA-based assays are used, sequences need to be transcribed. While in some assays regions are chosen that distinguish even closely related species (e.g. the 16S-23S rRNA intergenic spacer region) [50], other assays use sequences that recognise a broad range of strains specific to a body site (e.g. faecal strains) [49]. Since microbes can be abundantly present on a body site, good sensitivity may be obtained but also background or weak signals may occur for other body sites or non-human origins (animal samples or foods like yogurt) [49]. For that reason, qPCR assays may be useful [56].

4.8. Combining marker types

Since specific markers may be especially suited to identify a certain body fluid, assays have been developed that combine marker types. In a first approach, microbial markers for the identification of vaginal origin were combined with mRNA markers that mark other body fluids like blood, saliva, semen and menstrual secretion [74,81] or menstrual secretion only [110]. In a second approach, miRNA markers for either blood and saliva [96] or blood, semen and menstrual secretion [95] were combined with commercially available DNA profiling kits. In the latter publication, the DNA and RNA extracts from the same sample are separately amplified after which the products are combined prior to CE. The third combinatorial approach uses DNA methylation and microbial markers [63]. Bacterial markers known to occur in the oral and the vaginal cavity are used to mark saliva and vaginal mucosa/menstrual secretions. Semen has a clear distinct methylation pattern and is identified by four markers, while blood is inferred from the absence of bacterial markers and a methylation pattern distinct from semen. Although all these methods combine various strong markers, the systems may still present a challenge when compromised and mixed samples are involved.

5. Interpretation guidelines

Assays for mRNA, miRNA, DNA methylation or microbial markers all carry interpretational challenges when forensic stains are involved. Variations in genetic constitution (gender, ethnicity), physiology (age, pregnancy, disease), intake (nutrition, drugs) or environment (climate) can result in changed m(i)RNA levels, altered DNA methylation levels or (dis)appearance of a certain microbial species. As a solution, several markers per tissue can be used, which raises the question how to interpret results when only one marker presents a signal or informative value or when weak signals are obtained. Interpretation is also affected by the presence of multiple sources (same or other donor, see Section 3), which can reduce or nullify the discriminatory value of markers. To accommodate these features, interpretation strategies, models and guidelines have been developed, which are exemplified below.

For end-point RT-PCR mRNA assays two approaches have been proposed. The first approach is applied to multiplexes (both for body fluid and organ tissue inference) that target multiple cell types with at least two markers per cell type [46,111,112]. The method uses replicate analysis (preferably four amplifications) and an '$x = n/2$' scoring system per body fluid in which x is the number of observed and n the number of theoretically possible peaks considering all replicates. The results are scored in various categories: 'observed' if $x \geq n/2$, 'not observed' if $x = 0$ and 'sporadically observed' if $0 < x < n/2$. 'Sporadically observed' is a chosen terminology that effectively refers to 'no reliable interpretation possible'. For co-expressed cell types such as blood in menstrual secretion, 'and fits' is added when (sporadically) observed. All markers have the same weight in this approach, which is probably unrealistic. As an alternative approach, a numerical scoring method is described in which values are assigned to each of the used mRNA markers (five per body fluid, in individual multiplexes, single amplification each) based on correct and incorrect expression in samples of known origin [74].

From these numerical values a body fluid score is calculated and positive body fluid identification is given when the combined marker value is higher than a pre-determined threshold value. When cell types are 'observed' or 'above threshold', they are regarded present. For cell types that do not meet these requirements, a classification 'not present' may be a too strong statement and 'no indication for presence', 'presence cannot be excluded' or 'no statement' may be more appropriate [112].

Body fluid identification via the counting of mRNAs that have hybridised to a set of NanoString® probes has allowed the development of algorithms that generate a likelihood ratio (LR) comparing the likelihood of a given sample's profile with and without the presence of a particular fluid [76]. The probability distribution of gene expression in body fluids is modelled from datasets and this model is used to calculate maximum likelihood estimates for the levels of each body fluid in a sample. The model first described gene expression of a single fluid and was then extended to mixtures [76]. The algorithm is thus founded on a biologically reasonable model of gene expression and sound statistical principles. An LR of 100 was taken as the decision threshold for all body fluids types, but this threshold may be lowered (which increases the positive call rate) without generating false positives [76]. The system does not need normalisation from housekeeping genes (that were found to be non-uniformly expressed). Furthermore, this hybridisation-based assay allows both attenuation of markers that have very high expression levels (by including also unlabelled oligonucleotides) and enhancement of markers that have relatively low expression levels (by adding multiple probes that bind along that mRNA target) [76].

For miRNA markers, binary logistic regression has been applied to accurately predict the presence of menstrual secretion and other body fluids [88,89]. The C_T values obtained in miRNA RT-qPCR profiling assays were normalised using a reference miRNA gene and these ΔC_T values were plotted in 2D-scatterplots. Logistic regression (a statistic that describes the relationship between the probability of correctly identifying an outcome and a set of predictor variables) was applied as interpretation metrics. For the miRNA expression binomial probability theory was used, as numerical values cannot be predicted. The outcome is therefore binary which means that the unknown body fluid is for example menstrual secretion or non-menstrual [88,89].

The commercial DSI-semen assay that uses methylation markers for the identification of semen comes with an algorithm that presents a confidence level for the inference [98,113]. The assay uses a digestion with a methylation-sensitive restriction enzyme followed by multiplexed end-point PCR and CE. Eight loci are regarded: one for digestion-control, two as amplification-controls and five regarding semen identification with two loci methylated in non-semen tissues (and unmethylated in semen) and three loci specifically methylated in semen. Peak heights are used for data interpretation upon which the software aborts source identification when the amplification controls are inapt (either too high, too low or not sufficiently balanced), the restriction enzyme digestion is incomplete or when the probability of error is more than 0.001. The result read-out is then 'semen', 'non-semen' (with a probability of error value) or 'inconclusive' (without probability of error value). Mixtures of semen and non-semen body fluids may receive the read-out 'inconclusive', although for unbalanced mixtures 'semen' or 'non-semen' outputs were obtained as well [113].

Another approach builds around a multiplex system that analyses three origins (vaginal, oral and faecal) by two microbial species each [56]. The qPCR assay aims to identify the presence of vaginal fluid for which two aspects are used: (1) the C_T (cycle threshold) value that is achieved for one or both of the vaginal markers (using four categories $C_T < 30$, $30 < C_T < 35$, $C_T > 35$, no signal) and (2) whether signals for one or more of the other bacteria are obtained (using again four categories: no signal, $C_T > 35$, $30 < C_T < 35$, $C_T < 30$). The results are combined in a verbal scale that uses six categories regarding the presence of vaginal fluid: identification, highly probable, probable, neither identified nor excluded, not very probable, can be excluded [56]. No statement is made for saliva or faecal material [56].

Most of the above methods showed suitability when mixed samples were analysed [46,76,88,111–113]. The co-expression of cell types like blood with menstrual secretion makes it very challenging to recognise true mixtures of such two body fluids, whatever scoring procedure used. Context information, results of other evidentiary traces in the case and the corresponding DNA profiling results may be of assistance to a forensic scientist. Combining the results of DNA profiling and cell type inference holds the risk of 'association fallacy' when mixed samples are assessed: although it may be tempting to associate the cell type giving the strongest signals to the major donor in a DNA profile, this may be incorrect because not one donor donated one cell type or because cell type signals and DNA signals do not straightforwardly relate [114]. Correct association between donors and cell types is currently only possible when only gender specific body fluids and two donors of different genders are involved.

Table 3
Additional forensic applications for the various marker types.

Area	Forensic relevance	Marker type			
		mRNA	miRNA	Meth	Microbial
Molecular pathology	Post-mortem interval	x	x		x
	Cause of death, cardiac, drowning	x			x
	Ante- or post-mortem burn	x			
	Fatal hospital infection				x
Pharmacoepigenetics/behaviour	Response to drugs, inclination suicide		x	x	
Maternal blood	Blood of pregnant woman	x	x		
Dermal wounds	Dermal injury timing	x			
Epidermal layers	Distinguishing contact types (not successful)	x			
Decay ratio	Stain age	x			
Biological clock	Bloodstain deposition time of day		x		
Age	Donor age			x	
	Newborns	x			
Individualisation	Discrimination monozygotic twin individuals			x	
	Individualisation skin deposits				x
Geographical inference	Locality of soil				x
Authentication	Artificial DNA detection			x	
Parentage testing	Parent-of-origin allele			x	

6. Further applications

This review has focussed on cell type inference for which four marker types were described. Interestingly, the marker types have been implicated in other forensic uses as well, which are summarised in Table 3. Some subjects are more in the fundamental or exploratory phase with not yet clear markers such as pharmacoepigenetics in which the influence of epigenetics variants on drug response is studied [115] or behavioural epigenetics where for instance the association of suicide attempts and increased methylation is examined [116,117]. Other areas have retrieved functional markers or assays such as mRNA markers to infer dermal injury time (useful for case of violence and abuse) [118], microbial markers to infer soil locality, which may link soil found on evidentiary items (shoes of a suspect) with the soil at the crime scene [119–122] or methylation markers to determine the parent-of-origin allele, which is useful in paternity testing and especially the motherless cases [123,124]. Some publications report the failure to identify apt markers such as mRNA markers corresponding to various skin layers to distinguish different types of contact [59] or describe adverse results like on the individualisation of skin deposits through microbial markers [125–127].

Some subjects have the potential to be applicable to numerous forensic cases such as determining the age of the donor for which much progress is made with methylation markers [128–130] and the age of a stain. Regarding this deposition time, the decay rates of RNAs may be of use [131–134], although the influence of environmental conditions such as relative humidity, temperature, light, microbes in turnover processes may complicate the estimations. In contrast to assays based on haemoglobin turnover [135], RNA-based tools have the potential to not only apply to bloodstains. Other subjects may occur rarely in forensic practice like the authentication of human origin of DNA versus artificial DNA [136], the determination that blood originates from a woman at the time she was pregnant [137] or from a baby when it was a newborn [138]. The discrimination of monozygotic twins may not be a frequent forensic challenge but can be highly valuable for a specific case as both individuals may be acquitted if the perpetrator cannot be identified. Progress on this subject has not only been described for methylation markers [139,140] but also using the analysis of rare somatic single nucleotide changes that can be identified by ultra-deep-sequencing [141]. Also on the subject of deposition of a stain in the context of the biological clock (basically night or day deposition), other markers, in this case protein-based markers [142], outperform the miRNA markers [143].

In the context of molecular pathology, subjects include post-mortem interval determination via miRNAs [144–147], mRNAs [147–149] or microbial markers [150] and cause of death by cardiac disease via mRNA markers [151,152] or from drowning through microbial markers [30]. Microbial markers can also be useful to mark a fatal hospital infection [30]. Another subject is the diagnosis of ante mortem burns compared to post-mortem burns using an mRNA marker [153].

7. Concluding remark

This review has not expressed which marker type and which exact marker is best selected to infer a certain body fluid or tissue type. Marker type choice will be influenced by expertise and equipment in a forensic laboratory and new marker identifications will progress insight in the near future. Rather, the biological background and the accompanying interpretational challenges for each of the marker types were highlighted. Furthermore, the forensic relevance and scenarios that different body fluids and tissue types represent were described. Forensic cell type inference is an exciting field with much progress and developments. Importantly, molecular assays are increasingly applied to casework, which will provide valuable feedback and inspiration to further research.

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